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13. ABSTRACT (Maximum 200 Words)

This project aims to identify adult prostate stem cells, using tissue recombination techniques. To date, we have initiated studies utilizing mouse and human embryonic stem (ES) cells as outlined in the original statement of work. We have made progress towards directing differentiation of these ES cells into prostate lineages. We have shown pathologically and histologically that the resultant tissue recombinants have several characteristics of mouse and human prostate. Specifically, we have demonstrated tissue differentiated from human ES cells that have all the hallmarks of human fetal prostate. We are currently refining the technique in the hope of getting tissues that are immuno-positive for the unequivocal marker of mature prostate, within Australia and further findings will be presented in the coming months at several international meetings.

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INTRODUCTION

This project ultimately aims to identify adult stem cells in the prostate gland. It is well accepted that stem cells reside within the prostate epithelium, and it has been suggested that they are housed within the basal cell compartment. However, to date, prostate stem cells have not been isolated or characterized. We believe that the stem cells in the adult prostate have a major role to play in the initiation and progression of prostate carcinogenesis and so identifying these cells will have major implications in treating prostate cancer. We are employing a unique approach involving the use of mouse and human embryonic stem cells to track the differentiation of prostate epithelial cells to identify adult prostate stem cells.

BODY

Task 1: To generate prostatic ductal structures from ES cells using tissue recombination techniques (months 1-6).

We have completed all the experimental aims listed under task 1 in the first year of the project. We were able to establish protocols for pre-inducing mouse ES cells to form endoderm in a hanging drop culture system, generating embryoid bodies (EBs). These EBs are shown in **Figure 1**.

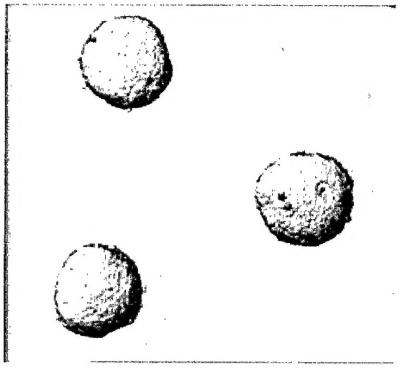

***Figure 1**: Images of three individual embryoid bodies formed from growing mouse ES cells in hanging drop cultures.*

In addition, we were able to utilize these EBs in tissue recombination experiments with both urogential mesenchyme and seminal vesicle. These tissues proved to be a suitable source of inductive instructive mesenchyme in that they directed differentiation of mouse ES cells into prostate-like tissue structures.

We were successful in grafting these recombinants under the kidney capsule to promote growth of prostate-like tissue. Tissue recombinants were grown in host animals for a period of 4 weeks. We compared these tissue recombs to grafts prepared from mouse ES cells alone, which resulted in large teratocarcinoma as expected (**Figure 2a**). In contrast, tissue recombinants composed of mouse ES cells and mouse SVM resulted in controlled growth of the tissue that was significantly smaller than the mouse ES cell controls (**Figure 2b**).

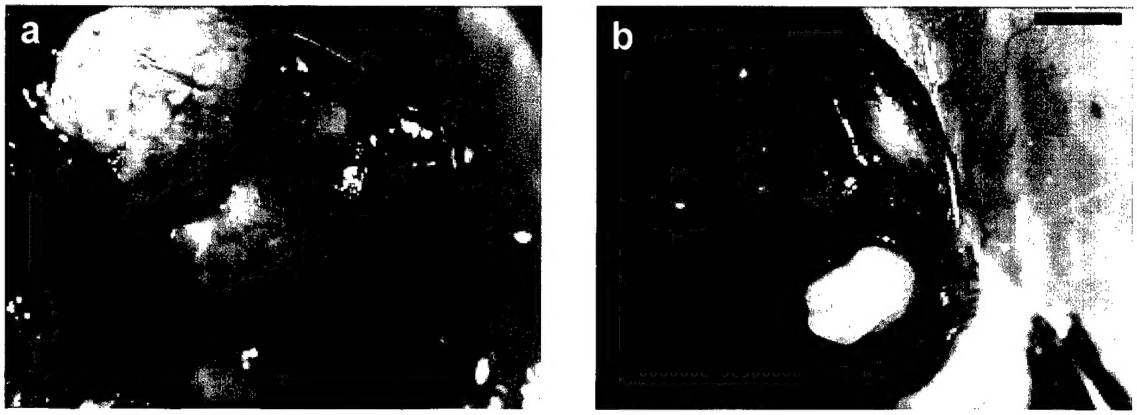

***Figure 2**: Images of grafts on the kidney capsule at the time of harvesting. **a.** Graft of mouse ES cells alone; the result is the formation of a large teratomcarcinoma. Rich vascularisation and multiple tissue types are evident. **b.** Graft of mouse ES cells with mouse SVM; the result is the formation of small grafts that appear to be fluid filled ductal structures, reminiscent of prostate-prostate recombinants. Bar = 2mm.*

We conducted extensive histological analysis of the grafts following collection from the host mice. The histology of the teratocarcinomas is shown in **Figure 3**. As the photomicrographs demonstrate, the teratocarcinomas were composed of tissue representative of all three germ layers (endoderm, ectoderm and mesoderm), resulting in a number of various tissue types identifiable throughout the tissue, proving that these cells are pluirpotent (**Figure 3a, b**). Although these teratocarcinoma's did contain epithelial ductal structures as marked by high molecular weight cytokeratins, these cells were not immuno-positive for the androgen receptor, which would be indicative of prostate-like tissue (**Figure 3c, d**).

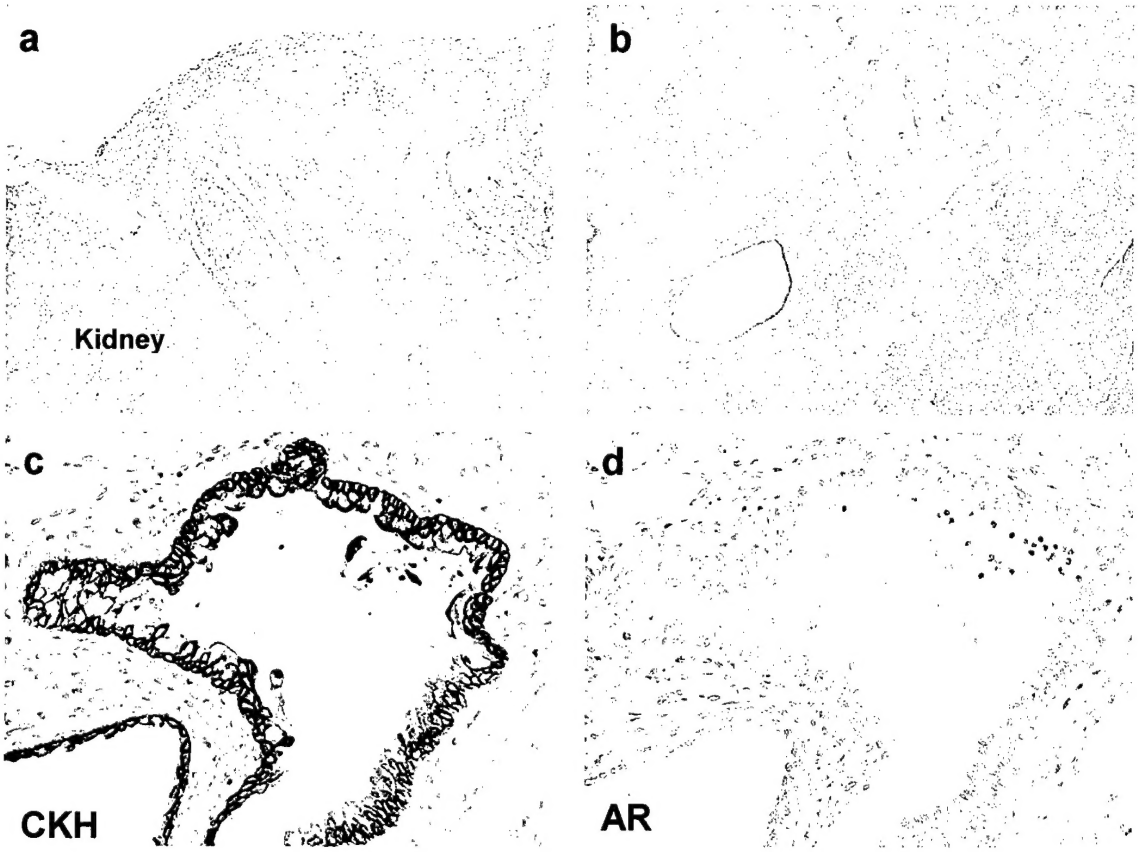

Figure 3: *Photomicrographs of tissue resulting from grafting mouse ES cells only in an immune-deficient male mouse for a period of 4 weeks.* ***a.*** ***b.*** *Haematoxylin and eosin stained sections,* ***c.*** *Immunolocalisation of high molecular weight cytokeratins (CKH; epithelial cell marker),* ***d.*** *Immunolocalisation of androgen receptor (AR).*

In contrast, tissue recombinants consisting of mouse ES cell/SVM grafts were histologically similar to prostate ductal structures (**Figure 4**). The graft consisted of ductal structures lined by epithelial cells surrounding lumens where secretory products were evident (**Figure 4A**). These epithelial cells were proven to have arisen from mouse ES cells by lacZ staining (**Figure 4B**; mouse ES cells were tagged with the βgal promoter). The epithelial cells were androgen receptor immuno-positive (**Figure 4B**) and high molecular weight cytokeratin immuno-positive (**Figure 4D**), characteristic of prostate tissue.

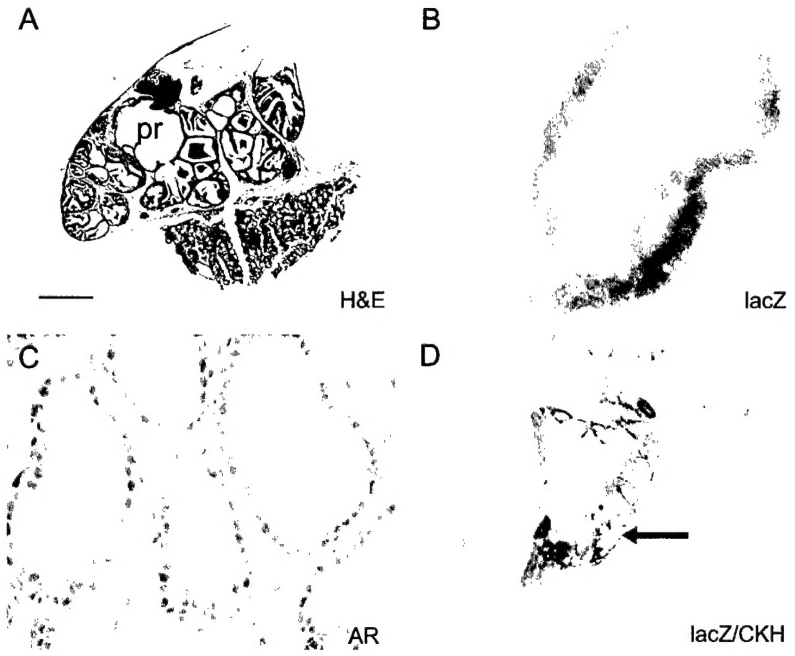

Figure 4**: Photomicrographs of tissue recombinants of mouse ES cells with mouse SVM.* ***a. *Haematoxylin and eosin staining.* ***b.*** *lacZ visualization.* ***c.*** *Immunolocalisation of androgen receptor (AR).* ***d.*** *Co-localisation of lacZ with immunolocalisation of high molecular weight cytokeratins (CKH, basal epithelial cell marker). Immuno-positive basal cells highlighted by arrow (→).*

This result was consistent and reproducible over multiple experiments. One difficulty we faced however, was finding a definitive marker for mouse prostate tissue. There are several markers that are characteristic of prostate tissue, but very few that are prostate-specific. Prostate specific antigen (PSA) is the gold standard in the field, but the antibody that is available does not recognize mouse prostate epithelial cells. Alternatively, Donjacour and Cunha (1993) developed an antibody against mouse prostatic protein secretion, named mDLP, that recognizes some mouse prostate lobes, but we could not get positive immunolabeling on our ES cell recombinants. Since we are using SVM and UGM to differentiate mouse ES cells, we were not certain of the analogous mouse prostate lobe that was being created and perhaps that was the problem with the staining.

Since there was no easy way around this problem (short of raising a new panel of antibodies to mouse prostate), we decided to move directly onto task 4, using human embryonic stem (hES) cells, since the PSA antibody was available and would definitely identify human prostate epithelium.

Task 2: To use these tissues for the isolation of sufficient numbers of basal and intermediate cell types from the epithelia, that can be tagged with appropriate markers prior to differentiation (months 6 – 18)

We have made some progress towards this task in anticipation of getting definitive prostate material from tissue recombination experiments. To date, we have generated two constructs that will be used to transfect mouse and/or human ES cells prior to recombination in preparation for cell sorting following the grafting period (task 3).

The first construct is the human keratin 18 gene promoter driving DsRed expression and the second is the human keratin 5 gene promoter driving GFP expression. These constructs will be available for transfection of ES cells at an appropriate time of the project.

Task 3: To determine which cell type is the prostatic stem cell of the epithelia (months 12-24)

No progress on this task to date. Once we get differentiation of ES cells into consistent, proven prostate tissue, we will embark on tracking prostate epithelial differentiation using the methods described in this task.

Task 4: To adopt the same strategies and techniques to prove the identity of human prostate stem cells using human ES cells (months 24-36).

Although we originally stated that we would not undertake these experiments until months 24-36 of the funding, we have initiated studies towards this task within the first year of funding. The main reason being our lack of appropriate mouse prostate markers. In addition, human and mouse embryonic stem cells grow under distinctly different culture conditions and therefore we needed time to adapt the recombination technique for hES cells.

Initially we were able to establish the growth of two hES cell lines in our laboratory; hES 2 and hES 4. These cells grow in colonies on a bed of mouse fibroblasts as shown in **Figure 5a**. During propagation, these colonies are cut into 'transfer pieces' as shown in **Figure 5b**. Part of these transfer pieces are used in the tissue recombination studies, in contrast to the embryoid bodies using mouse ES cells.

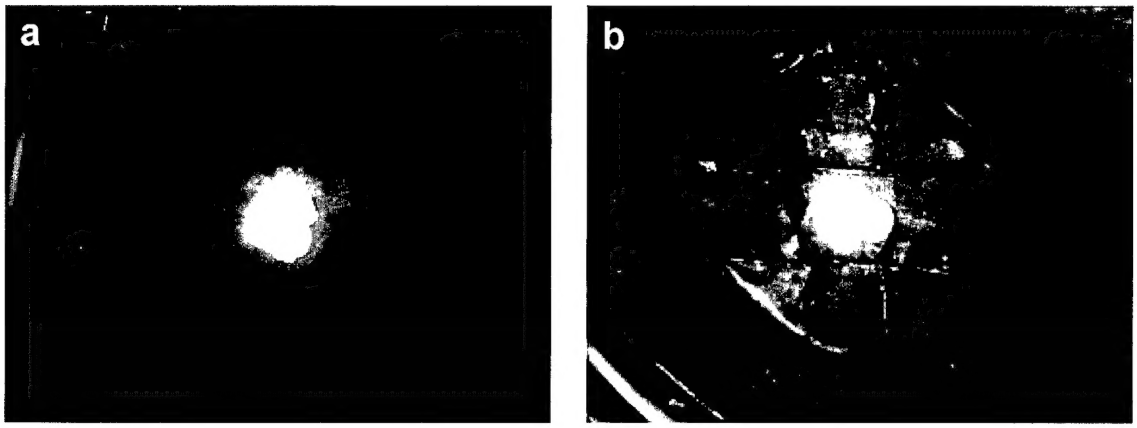

***Figure 5** –Human embryonic stem cell colonies. **a.** hES 2 colony growing on a mouse embryonic fibroblast layer. **b.** Transfer pieces of a hES 2 colony in preparation for tissue recombination experiments.*

Similar to mouse ES cells, we were successful in grafting tissue recombinants composed of hES cells and prostatic mesenchyme under the kidney capsule to promote growth of prostate-like grafts (**Figure 6**). Tissue recombinants were grown in host animals for a period of 4, 8 and 12 weeks. We compared these tissue recombinants to grafts prepared from hES cells alone, which resulted in large teratocarcinoma as expected (**Figure 6a**). In contrast, tissue recombinants composed of hES cells and prostatic mesenchyme (UGM or SVM) resulted in controlled growth of the tissue that was significantly smaller than the mouse ES cell controls (**Figure 6b**).

***Figure 6**: Images of grafts on the kidney capsule at the time of harvesting. **a.** Graft of human ES cells alone; the result is the formation of a large fluid-filled teratomcarcinoma. Rich vascularisation and multiple tissue types are evident. **b.** Graft of human ES cells with mouse SVM; the result is the formation of small grafts similar in size and appearance to prostate-prostate recombinants. Bar =1mm.*

Again, similar to mouse studies, we conducted extensive histological analysis of human ES cell recombinants. To date we have analyzed tissues from the 4 week and 8 week time points and the results are represented in **Figure 7**. After 4 weeks growth in the host animals, hES/SVM recombinants showed histology reminiscent of human fetal prostate (**Figure 7a-c**). Immature ductal structures were evident, with solid epithelial cords beginning to undergo canalization, surrounded by smooth muscle cells lining up around the ducts (**Figure 7a**). Immunolocalisation of cytokeratins 8 and 18 (**Figure 7b**) demonstrated that the ducts were composed of epithelial cells that were of human origin, proving that the ducts had arisen from the human ES cells and not contaminating mouse epithelial cells. In addition, both the epithelial cells and surrounding stromal cells were immunopositive for androgen receptors (**Figure 7c**). Collectively, these are all characteristic histological and pathological features of prostate tissue. However, immunolocalisation of PSA was negative, indicating that these tissues were prostate-like, but were not yet fully mature prostate epithelium (data not shown).

Therefore, we went on to examine the tissue that had been grafted for 8 weeks (**Figure 7d-f**). These tissues appeared more histologically mature than the previous time point, as expected. Ductal structures were present that were lined by pseudostratified columnar epithelial cells with significant lumen formation (**Figure 7d, e**). At higher power, it was evident that the secretory epithelial cells were beginning to secrete products that were visible in the cell cytoplasm (**Figure 7e**). In addition, both the epithelial cells and some of the surrounding stromal cells were immunopositive for androgen receptors (**Figure 7f**). Again, these are all characteristic histological and pathological features of prostate tissue. However, immunolocalisation of PSA was still negative (data not shown). We are currently in the process of leaving the tissues for 12 weeks in the host animals to allow more time for maturation of the tissue, and hopefully that will induce the onset of prostatic secretions and produce PSA-positive prostate epithelium.

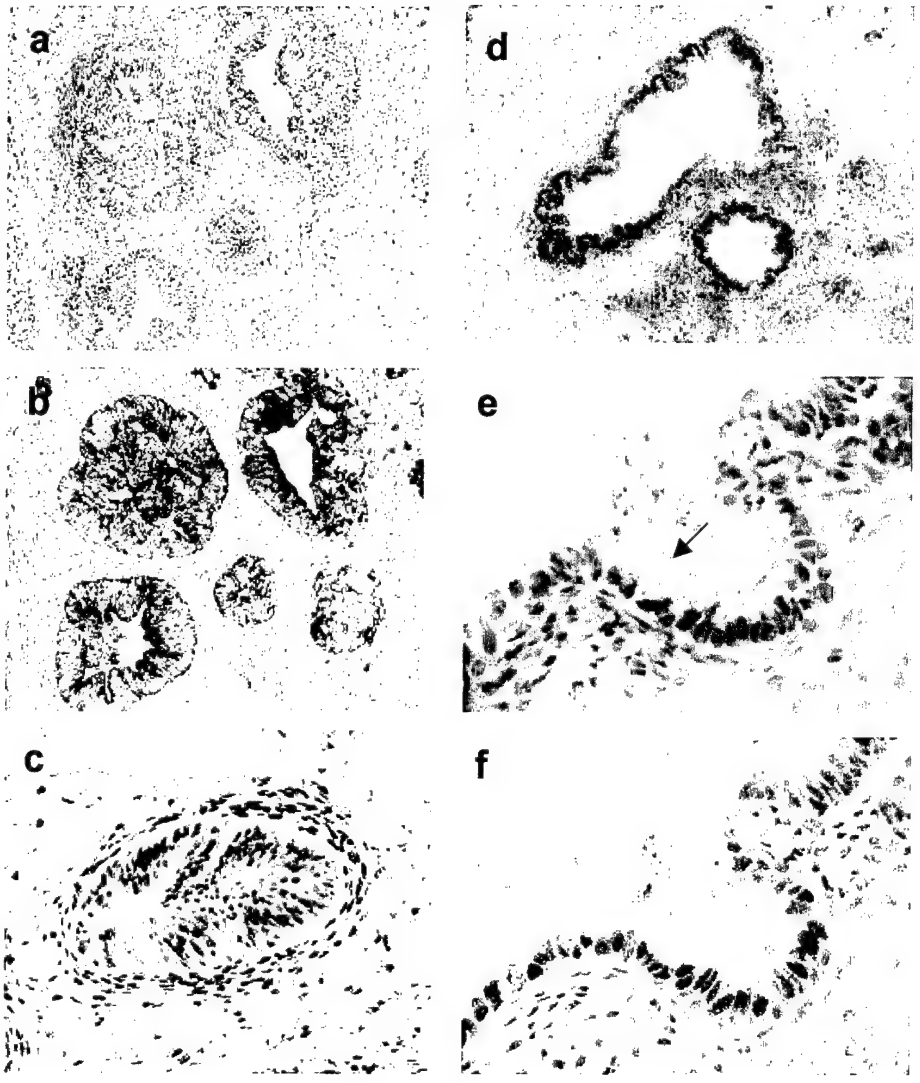

Figure 8 *– Histology of human ES cell recombinant following* ***a. b. c.*** *4 weeks and* ***d. e. f.*** *8 weeks growth in immune-deficient male mouse hosts.* ***a. d. e.*** *Haematoxylin and eosin stained sections.* ***b.*** *immunolocalisation of cytokeratin 8 and 18.* ***c. f.*** *Immunolocalisation of androgen receptor (AR). Arrow (→) shows secretory products in luminal cell cytoplasm.*

This time frame for maturation is comparable to previous reports using human epithelial cells (Hayward et al., 1998), so we are persevering with the technique until we get fully mature adult human prostate tissue before we move on to the next tasks for tagging and tracking the epithelia cell differentiation.

KEY RESEARCH ACCOMPLISHMENTS

List of key research accomplishments emanating from this research:

- Proved concept of controlled differentiation of ES cells using both SVM and UGM.
- Directed differentiated mouse embryonic stem cells into prostate-like tissues.
- Directed differentiated human embryonic stem cells into prostate-like tissues.
- Generated constructs for CK18 and CK 5 for future transfections.

REPORTABLE OUTCOMES

	Reportable outcomes that have resulted form this research:
Manuscripts	Nil.
Abstracts Presentations	1. **Jarred RA**, Wang H, Trounson AO, Risbridger GP (2003) Directed differentiation of human embryonic stem cells into prostate using tissue recombination. *The 1st National Stem Cell Centre Scientific Conference*, Melbourne, Australia. 2. Risbridger GP, **Jarred RA**, Wang H, Trounson AO (2003) All power to the prostatic stroma. *The 3rd National Prostate Cancer Symposium*, Melbourne, Australia.
Patents and licences	Provisional patent application filed March 2002 (663129) Full patent application filed March 2003 (663129)
Degrees obtained	Nil.
Development of cell lines	Nil.
Tissue or serum repositories	Nil.
Informatics such as databases and animal models	Nil.
Funding applied for based on this work supported by this award	Dr. Renea Jarred was awarded a Postdoctoral Traineeship Award from the Prostate Cancer Reseach Program, Department of Defense, based on preliminary observations from this work.
Employment or research opportunities applied for and/or received	Dr. Renea Jarred Postdoctoral Traineeship Award as described above. This position was awarded based on preliminary observations from this work to continue her studies in the laboratory of Professor Risbridger.

CONCLUSIONS

In summary, we have made significant progress towards directing differentiation of mouse and human ES cells into prostate lineages. We have shown pathologically and histologically that the resultant tissue recombinants have several characteristics of mouse and human prostate. Specifically, we have demonstrated tissue differentiated from human ES cells that have all the hallmarks of human fetal prostate. Future work will refine this technique and utilize the model to identify adult prostate stem cells.

This progress will have major implications to our understanding of prostate biology. In the same way that differentiation of embryonic stem cells into neurons has advanced the neurological field, these findings will have a major impact on urology. This research will further our understanding of the factors that induce prostate differentiation, that have eluded us until now.

Work from this project has led to the Postdoctoral Traineeship Award proposal that was granted to Dr. Renea Jarred to extend this model to investigate the differentiation of embryonic stem cells into prostate malignancy by carcinoma associated fibroblasts. Not only will these experiments advance our understanding of the initiating events that occur in prostate malignancy, but will provide us with information about the cell types of the prostate that should be targeted by disease therapies.

Overall, we believe the knowledge gained through this scientific project will greatly advance the understanding prostate biology and prostate disease.

REFERENCES

Donjacour, A. A., and Cunha, G. R. (1993). Assessment of prostatic protein secretion in tissue recombinants made of urogenital sinus mesenchyme and urothelium from normal or androgen-insensitive mice. *Endocrinology* **132,** 2342-50.

Hayward SW, Haughney PC, Rosen MA, Greulich KM, Weier HU, Dahiya R, Cunha GR. (1998) Interactions between adult human prostatic epithelium and rat urogenital sinus mesenchyme in a tissue recombination model. *Differentiation.* 63(3):131-40.

APPENDIX 1: ABSTRACT PRESENTATIONS

1. Invited symposium presentation given by Renea Jarred on Gail Risbridger's behalf at the 3rd National Prostate Cancer Symposium, Melbourne, Australia. This abstract is to be published in ANZ Journal of Surgery.

ALL POWER TO THE STROMA

Gail P. Risbridger[1], Renea A. Jarred[1], Hong Wang[1] and Alan Trounson[2]
[1]*Monash Institute of Reproduction and Development, Monash University, Clayton, Victoria, Australia*
[2]*National Stem Cell Centre, Notting Hill, Victoria, Australia*

Development, growth and cytodifferentiation of the prostate are androgen dependent and occur via reciprocal mesenchymal-epithelial interactions. Undifferentiated mesenchyme acts under the influence of testicular androgens inducing ductal morphogenesis and epithelial differentiation, while the epithelium reciprocally induces mesenchyme differentiation and morphological patterning of smooth muscle. In adulthood, normal epithelial growth and differentiation are regulated by reciprocal smooth muscle-epithelial interactions. In contrast, in prostate carcinogenesis, there is a break down in the stromal-epithelial interactions leading to de-differentiation of smooth muscle cells an apparent reversion of the stroma to an immature phenotype. McNeal termed this 're-awakening' of the prostatic stoma. However, further research into this phenomenon has been hampered by the lack of suitable models. Therefore, we sought to create a model of human prostate in the laboratory with which to further investigate these interactions to identify new cellular targets and test therapeutic compounds.

Embryonic prostate mesenchyme (urogenital mesenchyme, UGM or seminal vesicle mesenchyme, SVM) is known to act as a permissive and instructive inductor of epithelium originating from various germ layers, including endoderm, mesoderm and ectoderm (Cunha *et al.*, 1987). In this study we have attempted to use inducing mesenchyme to differentiate more primitive human epithelia; cells that have not yet committed to a germ layer, namely human embryonic stem (hES) cells.

Tissue recombination studies were conducted to induce differentiation of hES cells, under the instruction of prostatic mesenchyme. Recombinants were grafted under the kidney capsule of adult male immune-deficient mice for periods of up to 8 weeks, allowing them to develop in an adult male hormonal environment. Tissues were collected and prepared for pathological and immunohistochemical analysis.

The resultant grafts were histologically prostate-like, consisting of secretory glands lined by a pseudostratified columnar secretory epithelium, embedded in fibromuscular stroma. Using expression markers, we confirmed the epithelium was of human origin using a human-specific antibody raised to cytokeratins 8 & 18 (epithelial cell markers). In addition, the tissue was immuno-positive for androgen receptors, both in the stromal and epithelial cell types, consistent with prostate expression pattern.

We have provided evidence that prostate mesenchyme can induce differentiation of primitive epithelia, prior to commitment to germ layers. This study also provides the first demonstration of directed differentiation of human embryonic stem cells into prostate. We intend to use this model to investigate the cell lineage pathway in normal human prostate to definitively identify adult prostatic stem cells. We also intend to test the influence of tumour stroma to determine if the 're-awakened' stroma has the ability to induce malignant differentiation of primitive epithelia.

Reference: Cunha GR, Donjacour AA, Cooke PS, Mee S, Bigsby RM, Higgins SJ, Sugimura Y (1987) The Endocrinology and Developmental Biology of the Prostate. Endocrine Reviews 8(3):338-362.

2. Poster presentation given by Renea Jarred at The 1st National Stem Cell Centre Scientific Conference, Melbourne, Australia.

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DIRECTED DIFFERENTIATION OF HUMAN EMBRYONIC STEM CELLS INTO PROSTATE USING TISSUE RECOMBINATION

Renea A. Jarred[1], Hong Wang[1], Alan Trounson[2], Gail P. Risbridger[1]

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[2]*National Stem Cell Centre, Notting Hill, Victoria, Australia.*

The prostate gland is a male reproductive organ that is located at the base of the bladder and is often associated with disease upon aging, specifically prostate cancer and benign prostatic hyperplasia. In order to study the cell differentiation lineage associated with normal and diseased prostate, we used human embryonic stem (hES) cells as a tool to initially generate normal human prostate using tissue recombination. Tissue recombination (Denham *et al* 2003) utilizes the inductive and instructive potential of prostate mesenchyme to direct differentiation of hES cells in to prostate epithelial cells. Briefly, hES2 cells (ES Cell International; Reubinoff *et al.*, 2000) were recombined with neonatal mouse seminal vesicle mesenchyme (SVM) and grafted under the kidney capsule of adult male immune-deficient mice for periods of up to 8 weeks. The tissue was collected and prepared for pathological and immunohistochemical analysis. The resultant grafted tissue was prostate-like, consisting of secretory glands lined by a psuedostratified columnar secretory epithelium, embedded in fibromuscular stroma. Using expression markers, we confirmed the epithelium was of human origin using a human-specific antibody raised to cytokeratins 8 & 18 (epithelial cell markers). In addition, the tissue was immuno-positive for androgen receptors, both in the stromal and epithelial cell types, consistent with prostate expression pattern. This study provides the first evidence of directed differentiation of hES cells into human prostate tissue. The long term aims of this project are to label the hES cells prior to recombination in order to trace the differentiation pathway of human prostate epithelial cells in the hope of identifying adult human prostate stem cells.

Denham M, Trounson A, Mollard R. Respiratory lineage differentiation of embryonic stem cells in vitro. Keystone Symposia: From Stem Cells to Therapy, March 29 – April 2, 2003, Steamboat Springs, Colorado 2003

Reubinoff BE, Pera MF, Fong C-F, Trounson AO, Bongso A. Embryonic stem cell lines form human blastocysts: somatic differentiation in vitro. Nat Biotech 2000, 18: 399-404.